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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Representational difference analysis (RDA) was attempted on microdissected samples of human prostate cancer to identify areas of genomic homozygous deletion. Difference products were not reliably obtained with this procedure starting from DNA obtained from 5000 cells. When difference products were obtained, subsequent analysis showed that none corresponded to areas of homozygous deletion. To pursue an alternative strategy, oligonucleotide microarray analysis to identify genes that are down-regulated in malignant prostate tissue, procedures were evaluated that would allow the implementation of microarray analysis to microdissected prostate tissue samples. It was determined that approximately 10,000 cells is a reasonable compromise between the need to maximize input material and the time required to perform microdissection. Several amplification protocols and procedures were evaluated for efficacy of amplification and quality of oligonucleotide hybridization results. A working protocol is presented that will amplify mRNA from 10,000 cells to 70 ug after 2 rounds of amplification.

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Introduction

The goal of this idea grant was to apply a global genomic screening technique on clinical samples of human prostate cancer to discover novel tumor suppressor genes involved in the development of this disease. Microdissected tissue samples were used as starting points to overcome the confounding effects of non-neoplastic cells that surround prostate cancer cells in tissue, which can cause both false negative or false positive results, depending on the technique used. This report documents the unsuccessful attempts to apply representational difference analysis (RDA) and the RDA-derivative procedure differential subtraction chain (DSC) on microdissected samples. This report also details the optimization of protocols to use microdissected samples in an alternative global genomic screening strategy: oligonucleotide microarray transcript profiling.

Report

Phase 1, Tasks 1 and 2: Representational Difference Analysis (RDA) [1], or its variant form, Differential Subtraction Chain (DSC) [2] were performed a total of 20 times on microdissected samples, (or on known amounts of genomic DNA in trouble shooting procedures). Each microdissected sample contained approximately 5000 epithelial cells. In all but two attempts, the initial sample yielded amplicon populations with appropriate characteristics in gel electrophoresis analysis (See Figure 1 for examples).

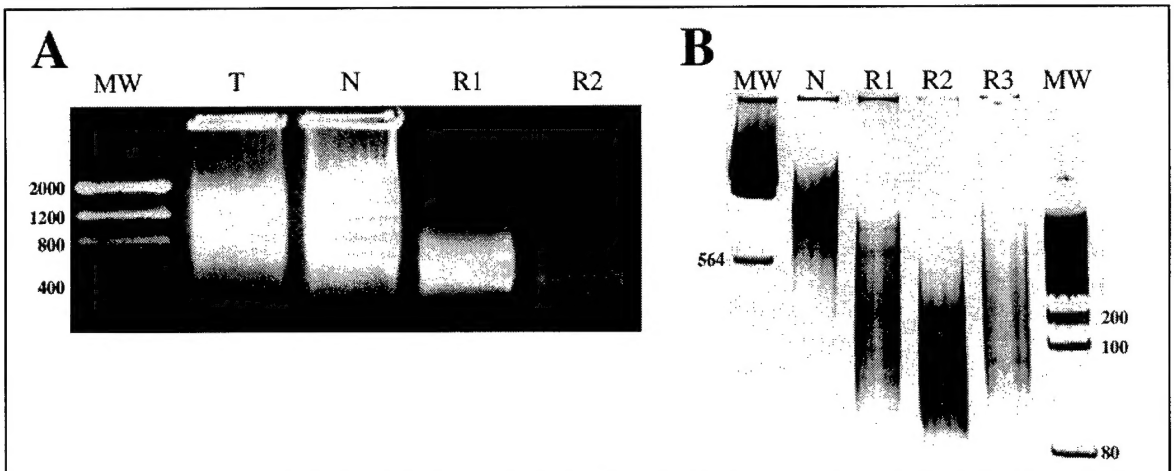

Figure 1: Examples of RDA derived from microdissected prostate cancer. PanelA: Gel electrophoresis (2% agarose – ethidium bromide stained). Initial amplicons derived from prostate cancer (T) and normal benign cells (N) show an expected result for the technique, with a smear of DNA with sizes ranging from 2000 base pairs downward. There is a marked simplification of the amplicon pool after one and two rounds of RDA (R1 and R2). However, no distinct bands are seen in the R2 difference products. Double strand DNA molecular weight markers (MW) are shown with their sizes in basepairs. Panel B: Gel electrophoresis (5% polyacrylamide –ethidium bromide stained-reverse image shown). As expected for the RDA procedure, there is a diminishment in size of the difference products through 3 rounds of RDA (R1-R3) as compared to the initial normal amplicon pool (N). In this RDA distinct bands were selected, although there was not complete suppression of a background smear of DNA.

The RDA/DSC procedures were then carried out to completion on these amplicon pools. The resultant products were analyzed by a combination of techniques:

1) Colony hybridization of cloned difference products using labeled driver and tester amplicon pools as probes. See Figure 2 for an example. This was one

method to screen clones present in normal DNA (the tester amplicons) but not present in cancer DNA (the driver amplicons), which would indicate the presence of a cancer-specific deletion.
2) Southern blot hybridization of amplicons using difference clones as probes. This method is a different way to detect true difference clones. See Figure 3 as an example.
3) DNA sequencing of recombinant clones of RDA or DSC difference products, with computer database Blast searches. This method is helpful in excluding the artifactual cloning of non-human sequences, and is required for the subsequent design of polymerase chain reaction (PCR) assays.

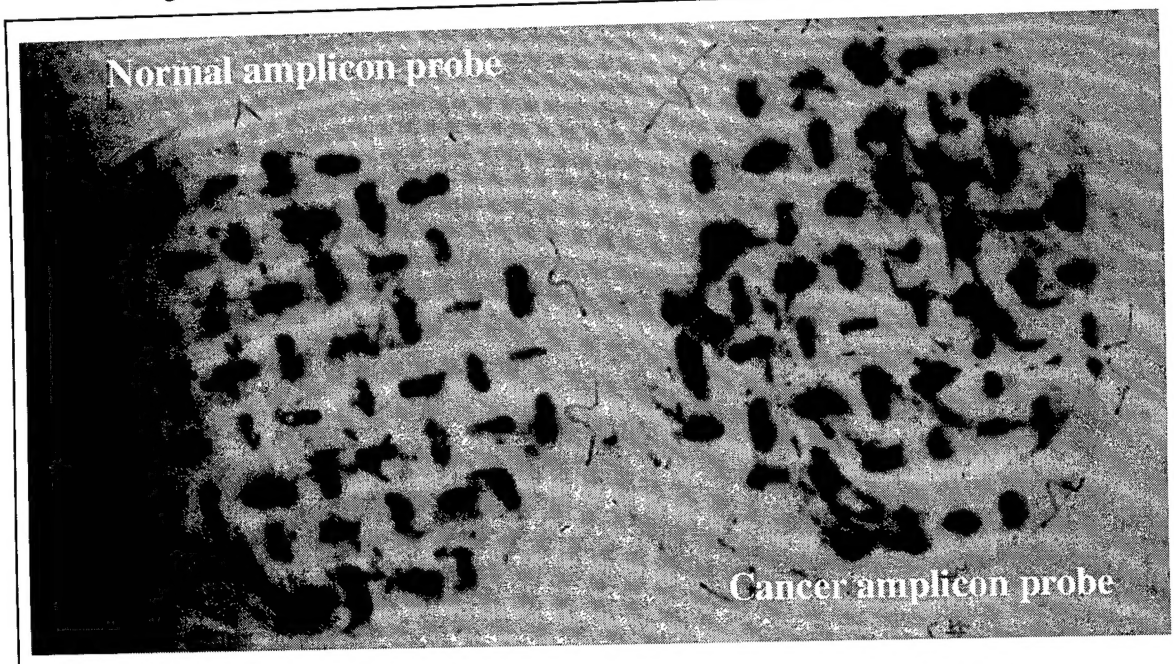

Figure 2: Example of colony lift screen of recombinant RDA difference product clones derived from microdissected prostate cancer. The duplicate colony lifts were probed with radioactively labeled amplicons derived from normal benign cells and from cancer cells of the same patient. A positive screen would result in a colony not hybridizing to the cancer amplicons, but hybridizing to the normal amplicons. No clone from this experiment represented a successful difference product.

Only four of the RDA/DSC procedures yielded discrete bands or marked simplification of the amplicon pool by the completion of the procedure which were adequate for subsequent analysis. A total of 95 recombinant clones from the 4 procedures (32, 8, 50 and 5, respectively) were screened for differential selection in the amplicon pools. Only one clone gave results in the screens indicating including characteristic signature of a deletion: absence of signal in the tumor amplicon pool and presence of signal in the normal amplicon pool (Figure 3).

Figure 3: Southern blot of RDA amplicons using difference product as a probe. Amplicons derived from microdissected prostate cancer ("driver") and normal cells ("tester") were subjected to gel electrophoresis and membrane transfer. A radioactive probe prepared from one of the difference products hybridized only to the normal tester population, indicating that it is absent from the cancer driver amplicon population.

To test whether this sequence was homozygously deleted in prostate cancer, a PCR assay was devised on unique clone sequences. PCR was performed on microdissected aliquots of the parent tumor. The results show that this sequence is not a target of homozygous deletion (Figure 4).

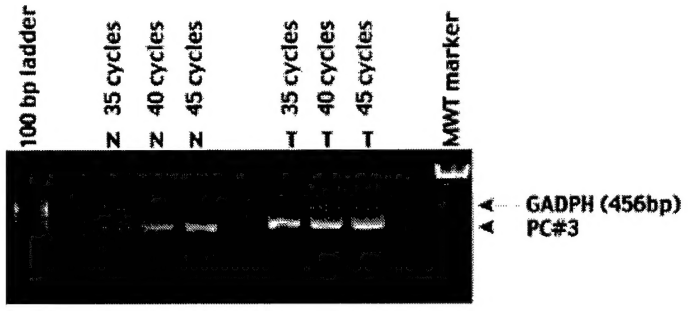

Figure 4: PCR assay testing for homozygous deletion of candidate RDA difference clone PC#3. DNA was obtained from microdissected aliquots of benign cells (N) and prostate cancer cells (T). In the PCR assays for tumor, a second set of primers were added (GAPDH) as a duplex control. The candidate DNA sequence (PC#3) amplifies as efficiently from the tumor cells as from benign cells, hence is not homozygously deleted in the tumor.

The RDA procedure is known to isolate restriction fragment length polymorphisms in areas of heterozygous deletion [3,4], and the results that we obtained for this clone (sequences absent from amplicon population, but present in parent genomic sequences) are consistent with that phenomenon. Theoretically, these procedure should have detected many more heterozygous deletions even if homozygous deletions were not present. To be certain that our tumor population did indeed contain heterozygous deletions and hence was representative of prostate cancer in general, we screened some of our prostate cancer samples for heterozygous deletions by traditional microsatellite loss of heterozygosity assays. The results showed that our tumor population did indeed contain heterozygous deletions, as most prostate cancers have been shown to develop (Figure 5). Hence, the lack of success in applying the RDA technique to microdissected samples was not due to an anomalous population of cancers being studied.

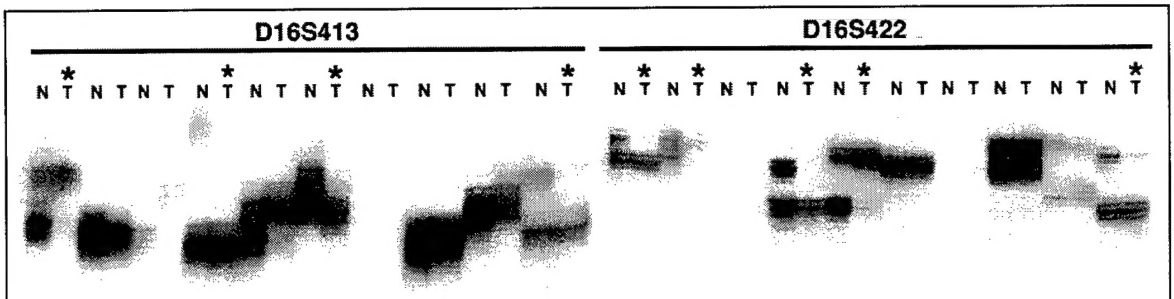

Figure 5: Microsatellite LOH assays on10 paired normal (N)/ prostate cancer (T) microdissected samples. Genetic deletion, as demonstrated by loss of one of two alleles in the cancer genomes in informative cases (asterix), was found in 4 cancers for the marker D16S413 and in 5 cancers for the marker D16S422.

During this time we checked our general RDA technique to be sure that it was working properly. We performed RDA with larger amounts (5ug) of genomic DNA which were identical, except for the spiking of the tester sample with bacteriophage lambda DNA (80 pg). The desired outcome is the isolation of only lambda DNA sequences from the tester population. Our results show that if we start from 5 ug of genomic DNA spiked with 80 pg of λ bacteriophage DNA, we can achieve efficient and complete isolation of the spiked DNA after just 2 rounds of RDA (Figure 6), hence the general procedure was working.

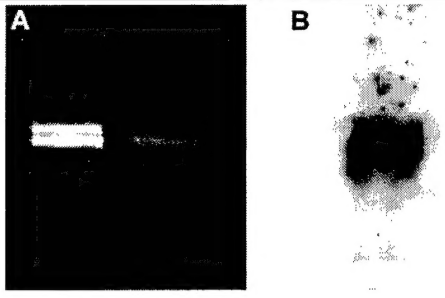

Figure 6: Results of control RDA. An RDA was performed using 5 ug bulk genomic human DNA spiked with 80 pg bacteriophage I DNA (6 oorders of magnitude difference). Panel A: Gel electrophoresis of difference products shows a marked reduction of complexity of the amplicon pool after one round of RDA (left lane), with a few sequences strongly selected. After two rounds of RDA (right lane) there is only one strongly-selected sequence. Panel B: Southern blot of round 2 difference product probed with radioactively labeled bacteriophage I DNA. The strong hybridization of the difference product shows that the RDA procedure successfully isolated a minute difference between 2 complex genomes, with at least a millionfold enrichment of the difference product.

However, since we had been unable to efficiently isolate difference products from microdissected tissues that represented deleted genomic regions by the end of the first 30 month phase, we concluded that RDA-type of technologies do not efficiently detect deletions from the limiting amounts of genomic DNA present in microdissected tissue samples.

During the course of these experiments a new technology had been developed for global screening of gene expression profiling: microarrays. The detection of substancial downregulation of gene expression is one characteristic of tumor suppressor genes, and we proposed to investigate the use of this technology on clinical specimens of prostate cancer as an alternative. This was proposed in our annual summary (September 1999) and was approved. In an effort not supported by our DAMD grant, we had supplied bulk tissue samples (not microdissected) of prostate cancer to a collaborator for gene expression analysis on Affymetrix GeneChips (oligonucleotide microarrays). This collaborative effort proved successful in identifying overexpressed genes [5], and showed that the tissue samples that we collected contained RNA with good integrity. In support of the effort for this grant, we used the same data set to screen for genes significantly

down regulated in an effort to find potential tumor suppressor genes. One of the down regulated genes was Mxi1 (OMIM 600020). In a panel of 25 tumors, the raw hybridization score from Affymetrix GeneChip analysis was 916 and in a panel of 9 normal prostate tissues, the raw hybridization score was 1513. From annotated data, Mxi1 is an obvious candidate for tumor suppression, as it is a negative regulator of the myc oncoprotein. The hypothesis generated was that down regulation of Mxi1 in prostate cancer cells led to increased activity of myc, the result of which would be increased cell proliferation. The first step was to devise a validation assay of the microarray data. We obtained a commercial antibody to Mxi1 (mouse monoclonal M51920, Transduction Laboratories) and performed immunohistochemistry on a number of histologic sections of primary prostate cancer. Figure 7 shows a representative example.

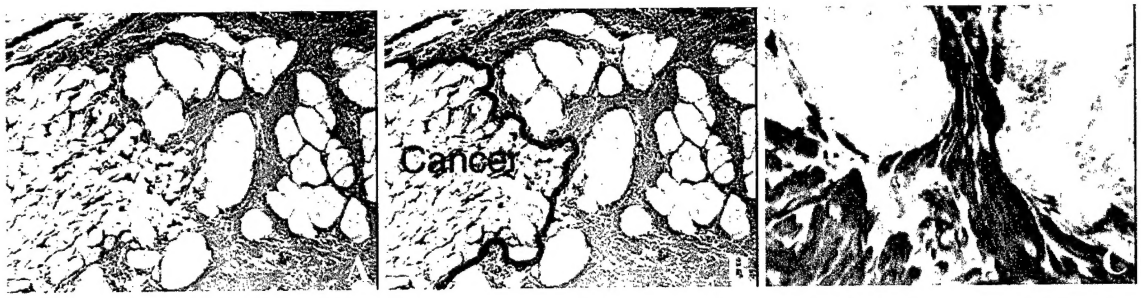

Figure 7: Example of Mxi1 immunohistochemistry in prostate cancer. Positive staining (brown color) is seen in prostatic stroma, not in prostate cancer cells or in normal prostate epithelium. This case is also illustrative of how, in general, areas of prostate cancer contain less stroma than areas of benign glands. A) Original magnification x 20. B) same figure as in A, showing area of cancer. C) Original magnification x 200.

What we found is that Mxi1 is normally expressed in the stroma of prostate and not in the epithelium of prostate (benign or malignant). Since bulk, non-microdissected samples of prostate cancer and normal prostate epithelium had been used, the samples of prostate cancer (which contain less stroma than normal prostate as a percent of tissue) resulted in a lower level of Mxi1 as a result of artifact of tissue architecture, rather than reflecting gene expression changes within prostate epithelium as a result of neoplastic transformation. This result reaffirmed the requirement for microdissection to detect specific losses of molecular components of prostate cancer. Most tumor suppressor genes found to date have been regulatory genes that are not expressed in normal cells at high levels. In order to detect subtle decreases in tumor suppressor gene transcription (as opposed to large increase in transcription that accompanies many oncogene activation events), it would appear that the confounding effects of contaminating non-neoplastic cells in tumor samples needs to be eliminated.

In order to use RNA from microdissected tissue, we first had to verify that RNA of appropriate quality could be obtained histologic preparations. We did so, by performing the initial histologic preparations on cryostat sections on a panel of prostate cancer samples, isolating the RNA, and performing reverse transcriptase assays for a panel of housekeeping genes, including a low abundance transcript (DNA polymerase epsilon). Our results showed that all the test genes could be detected, with at least 1700 bp of message length intact (Figure 8). RNA quality assessed by these methods was good after microdissection (Figure 9) and we validated that differences in transcription between

cancer and normal cells could be detected in RNA populations derived from microdissected material (Figure 10).

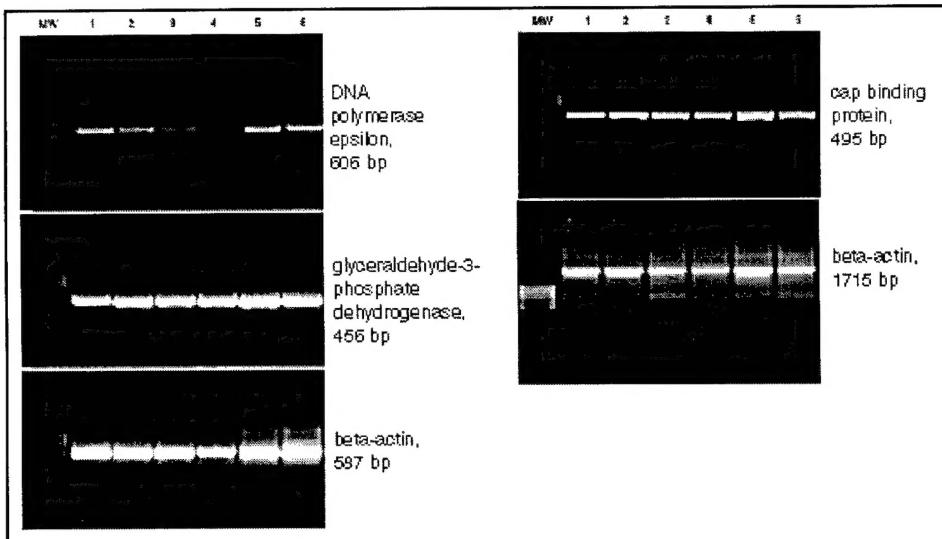

Figure 8. RT-PCR of housekeeping genes. The same 6 RNA samples as in Fig. 1 were used in RT-PCR assays of a panel of housekeeping genes that are known to be expressed at different levels in most mammalian cells. The results of the RT-PCR assays are shown in separate panels, with the name of the target genes to the right of each panel, as well as the size of the PCR product in basepairs (bp). 40 PCR cycles were used. The first lane (MW) in each panel contain molecular weight markers 100-1000 bp in 100 bp increments. The RT-PCR products are shown in the next 6 lanes. RT minus controls (not shown) had no PCR products, indicating that genomic DNA did not contaminate the RNA preps.

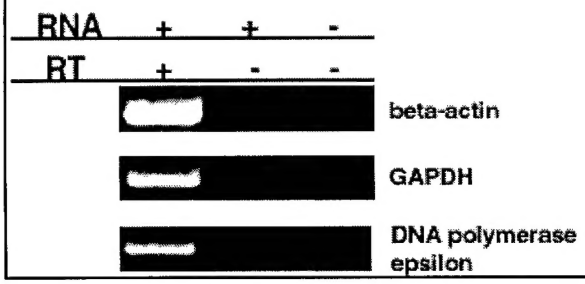

Figure 9. RT-PCR of a microdissected prostate cancer sample 5000 cells were microdissected from a 14 uM histologic section and subjected to RNA isolation. One aliquot was treated with reverse transcriptase (RT) while the other was not. Samples corresponding to approximately 500 cell input were then subjected to PCR for target housekeeping genes. The left-hand lanes show that the aliquots contain RNA for an abundant transcript (beta-actin), a moderately expressed transcript (GAPDH) and a low abundance transcript (DNA polymerase epsilon). No genomic DNA contaminating the RNA preps is detected (middle lanes). Negative controls for PCR contamination are shown in the right-hand lanes.

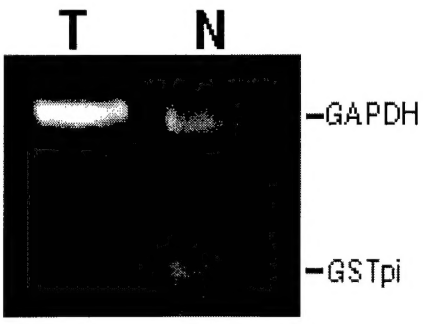

Figure 10 . Differential gene expression of GSTp identified in semiquantitative RT-PCR amplifications of microdissected prostate tissue. The figure shows a gel electrophoresis of duplex RT-PCR products. The upper product corresponds to GAPDH (456 bp) which serves as a relative control for the amount of input RNA. the lower band corresponds to GSTp(150 bp), an enzyme present in benign prostate epithelium, but which is known to be down-regulated in most prostatic carcinomas. The T lane corresponds to 250 prostate cancer cell equivalents assayed. the N lane corresponds to 250 cell equivalents of benign prostate epithelium obtained from the same patient. Although in this example the normal sample did not amplify as well as the tumor sample, the GSTp product is clearly absent from the tumor sample.

With the methodology in place to isolate good quality RNA from histologic sections, we turned our attention to microdissection. Because of the complex architecture of invasive

prostate cancer, we had previously shown to our satisfaction that manual microdissection techniques are inadequate for the precision required to obtain pure representations of prostate cancer (Figure 11).

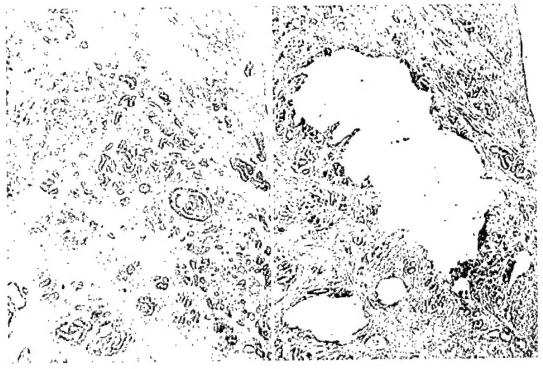

Figure 11: Manual microdissection of prostate cancer. Left panel: H&E-stained histologic section showing prostate cancer glands surrounded by benign stromal cells. Right panel: section after manual microdissection. The precision with hand-held instruments is not sufficient to separate cancer cells from intervening stromal cells.

Of the available laser-assisted microdissection technologies, in our hands the UV laser-assisted microdissection is superior in reproducible capture of pure prostate cancer representations (Figure 12).

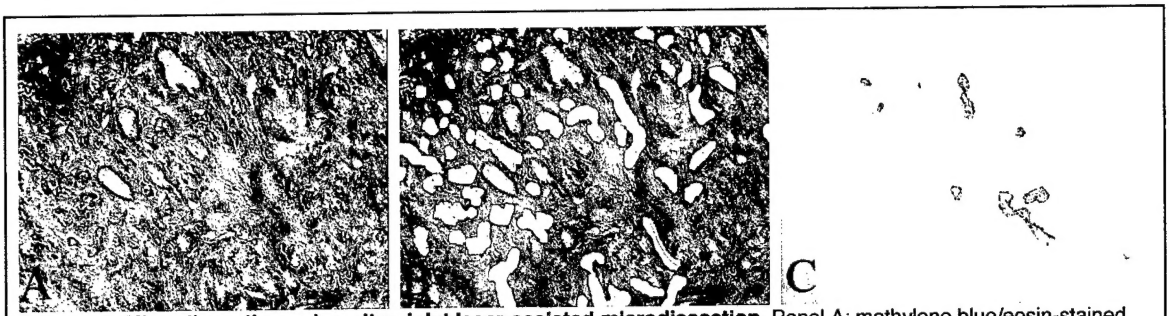

Figure 12: Microdissection using ultraviolet laser-assisted microdissection. Panel A: methylene blue/eosin-stained histologic section showing prostate cancer glands surrounded by benign stromal cells. Panel B: After microdissection, the section shows that cancer cells have been selectively removed, with the intervening stromal cells remaining. Panel C: An area of a microcentrifuge tube containing some of the microdissected proatate cancer glands.

Using the UV laser microdissection system from Leica Corp., we determined that the upper range of cell number that can be practically captured is 10,000, which requires approximately one full day per sample, which is up to four times as fast as using the hydraulic micromanipulator apparatus employed in the RDA studies. We estimated that this represents 100 ng of total RNA, which is equivalent to 1-5 ng of mRNA. The minimal amount of biotin-labeled cRNA required for reproducible results on an Affymetrix GeneChip is 10 ug. To overcome this discrepancy we needed a reliable procedure that would linearly amplify 100 ng of total RNA to at least 10 ug of cRNA. We rejected the use of PCR-based approaches due to the known non-linear amplification properties of this procedure which would skew the transcriptional profiling results. We began from a published procedure from the Eberwine laboratory [6,7], for linear RNA amplification (called aRNA) that entails the use of multiple rounds of cDNA synthesis and in vitro RNA transcription. One early problem encountered with the use of the second and third rounds of cDNA synthesis inherit to this technique requires the use of degenerate random oligonucleotides, that during hybridization, eliminate 5' transcript sequences. During our workup of these procedures, we therefore tried a 3' tailing

technique based on strand switch technology [8] (which we called linear amplification of strand switch cDNA or LASS cDNA) to provide a sequence-specific anchor for hybridization during the second and third rounds of cDNA synthesis. We also tried several variations on published techniques from the Eberwine and Watson laboratories[9]. A brief synopsis of our results follows:

1) 3' tailing procedures did not in our hands yield true representations of the beginning RNA populations
2) 3 rounds of aRNA resulted in RNA transcripts too truncated in 5' sequences to be detected on Affymetrix GeneChips
3) 2 rounds of aRNA resulted in acceptable transcript lengths, but required absolutely optimal conditions to yield sufficient transcript for microarray analysis.
4) For microdissected samples, standard GITC buffer RNA extraction and alcohol precipitation (Stratagene MicroRNA kit) is superior, in terms of RNA yield, to column-based RNA isolation procedures.

Table 1 details our procedural trials and optimization for these complex multi-step procedures. The analysis methods included a fluorescence-based method for RNA concentration/quantity determination (Ribogreen), RT-PCR for the assay of specific housekeeping genes within the RNA population, gel electrophoresis to determine the size distribution of the RNA population, Affymetrix GeneChip microarray analysis, and in the later stages, use of a microcapillary electrophoresis system (Agilent Bioanalyzer) to determine both size and quantity of the RNA population.

Table 1: RNA amplification optimization

Date	Experiment	Analysis Method	Result
2/22/00	compare cDNA synthesis using standard oligo-dT versus oligo-dT-T7 primer for RNA amplification	RT-PCR	successful synthesis using dT-T7 primer, but performance for low frequency transcripts poor
2/28/00	comparison of Trizol extraction versus Stratagene MicroRNA isolation kit for extraction from stained sections	RT-PCR	genomic DNA contamination significantly lower in Stratagene preps compared to Trizol; Stratagene demonstrated recovery of both long and low-expression test transcripts
3/13/00	tests of aRNA synthesis from microdissected prostate tissue using our RNA extraction/cDNA synthesis and Eberwine method for aRNA synthesis	RT-PCR	amplified RNA from 50 and 200 cell aliquots not demonstrated
	re-test of aRNA synthesis with modifications-cDNA purification using Qiagen PCR purification kit, alternate dT-T7 oligo, removal of S1 nuclease digestion	RT-PCR	positive RT-PCR tests for 50 and 200 cell aliquots, but pattern of results suggests that genomic DNA may still be present
4/11/00	tests of alternate DNase treatment (Ambion DNA-free kit) to eliminate Exonuclease III and additional PCI extractions	RT-PCR	effectiveness of Ambion kit demonstrated, but protocol needs to be modified for downstream use in aRNA procedure
4/19/00	tests of performance of Ambion kit in aRNA synthesis procedure with microdissected material	RT-PCR	cDNA synthesis weak
4/21/00	evaluation of Eberwine AMV-RT cDNA synthesis versus our standard Superscript II cDNA synthesis	RT-PCR	no cDNA observed from 500 cell equivalents
4/26/00	aRNA procedure using AMV cDNA synthesis and Qiagen PCR purification kit for cDNA isolation; vary dT-T7 concentration	RT-PCR	mRNA weakly detected for one of two transcripts tested after one round of aRNA in one of four samples

5/2/00	direct comparison of cDNA synthesis using AMV method versus SuperScript II procedure and dT-T7 oligo required for aRNA procedure on microdissected material	RT-PCR	test transcripts recovered from 500 cell aliquots in each test. AMV reverse transcriptase less efficient than SuperScript II in all tests and significant amount of product not full length
5/8/02	determination of product size distribution for amplified RNA prepared with SuperScript II protocol	radiolabeled RNA electrophoresis	some labeled RNA produced, but size distribution predominantly low molecular weight products; indicates poor quality RNA, cDNA not full-length, or T7 synthesis not full-length
5/16/00	examination of RiboGreen fluorometric assay as method for quantitative analysis of RNA isolated from microdissections and downstream aRNA synthesis	RiboGreen Assay	method is sufficiently sensitive for assay of RNA recovered from microdissected material and amplified material; quantity of total RNA obtained from microdissections varies considerably and is not an appropriate input for comparing downstream procedures;
5/22/00	preparation and characterization of high-quality RNA isolate from cell line	RiboGreen Assay, RT-PCR	purpose to provide a controlled RNA substrate for optimization of aRNA technique
5/24/00	aRNA procedure using SuperScript II protocol and spin-column cDNA purification	RiboGreen Assay	observed approximately 0.3 ng of cRNA from 50 and 200 cell equivalents; 0.03 ng from 500 and 0.15 ng from 5000 cell equivalents
6/1/00	pilot experiments on linear amplification strand switch RNA amplification technique (LASS)	RiboGreen Assay	using 2.5 (500 cell equiv) and 250 ng (500 cell equiv) total RNA template yields of cRNA were: one round aRNA 0.2 ng & 0.8 ng; one round LASS 18.9 ng & 25.9 ng; two rounds of LASS 3.9 ng & 12.6 ng
6/7/00	evaluation of Epicentre T7 Ampliscribe kit performance	RiboGreen Assay	100 ng of linear T7 template (PCR product from a commercial control template) amplifies approximately 80-fold using this kit
6/9/02	comparison of Epicentre Ampliscribe T7 kit to a homemade recipe using Ambion T7 enzyme	RiboGreen Assay	Ampliscribe kit amplified 80-fold versus Ambion enzyme procedure amplified only 10-fold using a linear T7 control template
6/12/00	titration of nucleotide concentrations and reaction volumes for Ampliscribe kit	RiboGreen Assay	Standard 20 µL reaction conditions produced 4 to 10-fold more RNA from 100 ng T7 control template when compared to all modifications tested
6/14/00	additional tests of T7 RNA polymerases from Epicentre and Ambion	RiboGreen Assay	using 100 ng of T7 control template, standard Epicentre Ampliscribe conditions produced 25-35 µg of RNA; standalone T7 enzymes failed over a broad range of reaction conditions
6/16/00	tests of cDNA synthesis and T7 RNA amplification using Epicentre Ampliscribe kit and increased input template to 5000 cells (microdissected prostate)	RT-PCR; radiolabeled RNA gel	RT-PCR results show that cDNA synthesis successful for all but longest transcripts tested; RNA produced by amplification covers a range of sizes, but is concentrated at and below 1500 bp
6/28/00	evaluation of alternative T7 synthesis buffer compositions (derived from various literature sources)	RiboGreen Assay	utilizing a T7 control template, alternative Tris and HEPES-based buffer systems produced 10-fold less cRNA than standard Epicentre Ampliscribe conditions; significant dependence on enzyme concentration noted

6/30/00	titration of T7 enzyme concentration in both Tris and HEPES-based buffer systems	RiboGreen Assay	highest yields (6 µg) were reached at 1000 U in Tris buffer and 2000 U in HEPES buffer; these are about half of what the Epicentre kit produces; cost compares favorably
7/6/00	comparison of input preparation methods for T7 synthesis systems	RiboGreen Assay	using T7 control template, alcohol precipitation of template before reconstitution in T7 reactions effectively prevented synthesis
7/13/00	tests of alternate Microcon (Ambion) purification of cDNA prior to RNA synthesis	N/A	multiple parallel tests gave widely varying volumes of eluate; inconsistency and inability to reliably concentrate to volume required for RNA synthesis without further treatment is a concern
7/25/00	modified LASS procedure using modifications from aRNA tests (DNase treatment; Epicentre Ampliscribe kits; Microcon cDNA isolation)	RiboGreen Assay	using stock RNA prep as input, 50 ng and 100 ng input into the LASS procedure gave no RNA output after 1 and 2 rounds; review of Microcon specifications suggests MW cutoff of YM-50 better suited to retaining lower MW RNA
8/23/00	modified LASS procedure using YM-50 Microcon	RiboGreen Assay	YM-50 holdup volume is too high to use without additional concentration; incorporate Speed-Vac step; no RNA detected after 1 or 2 rounds of LASS
8/28/00	LASS procedure with modifications to eliminate Speed-Vac	RiboGreen Assay	column holdup volumes excessive, and time to evaporate to usable volume compromises efficiency of procedure; use of heat to drive off water considered non-optimal; no RNA production detected; subsequent verification of Microcon performance showed that no
9/1/00	LASS procedure using PCI extraction/alcohol precipitation for cDNA isolations	RiboGreen Assay	32 µg cRNA obtained after first amplification with 100 ng total RNA template; 15 µg cRNA obtained with 500 ng total RNA template; possible interferences with RiboGreen assay noted for direct assays
	LASS procedure	RiboGreen Assay	designed to evaluate potentially interfering species in RiboGreen assay, demonstrated that purification of RNA prior to assay or use of mock reaction controls is imperative in LASS procedure (not an issue with aRNA due to differences in protocol)
9/15/00	LASS procedure; switch to ammonium salts for alcohol precipitations	radiolabeled nucleotide incorporation assay; RNA gel electrophoresis	incorporation corresponds to approx. 500 ng cDNA from 100 ng total RNA starting material; cRNA size distribution falls mostly below 2000 bp; subsequent evaluation of dT-T7 primer sequences used in LASS procedure were not designed correctly (failure assure
10/19/00	evaluation of aRNA procedure from Ohyama [10] and modified 3-round LASS procedure	RiboGreen Assay; gel electrophoresis	aRNA protocol used Microcon and cDNA could not be recovered due to excessive holdup volume; LASS results and mock reaction controls both identical, indicating that procedure failed; this result was verified by electrophoresis of reserved cDNA which only s

11/14/00	replicate LASS procedure using a no-template control	Ribogreen Assay	no RNA detected at all template input levels
	LASS procedure using high concentrations of template	RiboGreen Assay	using approx. 50 µg total RNA from a cell line, LASS procedure produced less product than theoretically present as message in the oroginal sample
1/19/01	tests of modified LASS procedure using Qiagen spin-columns and Novartis modified Eberwine protocol	RiboGreen Assay	to keep parity, Eberwine used 3 rounds of amplification; from 100 ng total RNA template, Eberwine gave 13.9 µg cRNA and LASS 0.4 µg cRNA
	modified Eberwine aRNA protocol tests using 10,000 microdissected prostate cells; parllel tests using two different types of Qiagen spin-columns for cDNA purification	RiboGreen Assay	after 2 rounds of T7 amplification, roughly equivalent yields of 1 µg cRNA for each procedure with mini format slightly better. This is still at least 1 order of magnitude too low for use in our application
2/6/01	tests to determine if RNA successfully obtained from prostate tissue sample microdissected using Leica LMD	RT-PCR	test successful for low MW GAPDH and Actin transcripts
	tests of modified Eberwine protocol using 10,000 microdissected prostate cells; compare Ambion MegaScript T7 kit to Ampliscribe Kit	RiboGreen Assay	both methods successfully produced nearly identical amounts (20-21 µg) of cRNA after 2 rounds of amplification
3/1/01	replicate aRNA samples using modified Eberwine procedure; 5 ng (500 cell equiv) and 100 ng (10,000 cell equiv) through 2 rounds of T7 amplification	Ribogreen Assay	0.3-0.4 ng cRNA yields for 5 ng template; 8 µg cRNA yield for 100 ng; deemed sufficiently high for attempted microarray procedure
3/9/01	replicate procedure using same conditions as above	Affymetrix GeneChip	No specific transcripts were present in the amplified material
4/6/01	evaluation of alternative aRNA procedures using modifications suggested by Baugh [11] and a modified LASS procedure incorporating a tailing reaction	RiboGreen Assay	after 2 rounds of T7 amplification using 10,000 cell equiv. input- "standard" aRNA gave 5 µg, tailing LASS gave 30 ng, Baugh modifications gave 130 ng
4/26/01	demonstration of efficiency of tailing reactions using 100 ng and 1 µg of RNA template	radiolabeled RNA gel electrophoresis	results inconclusive; too much unincorporated nucleotide; will need to change procedure to remove
5/2/01	tests using alternate priming strategy to trap for 3-prime products (T7 at 3-prime instead of 5-prime end)	radiolabeled RNA gel electrophoresis	method is working; labeled RNA products running in a smear; majority at or below 2 kB
5/14/01	tests of new T7 RNA polymerase product (Promega) versus Ampliscribe kit	radiolabeled RNA gel electrophoresis	change suggested due to suggested incompatibilities of Epicentre reagents with radiolabeling that we are doing; Ampliscribe significantly better than Promega enzyme using our LASS method
5/16/01	modified tailed LASS for quantitation	RiboGreen Assay	80 ng cRNA yield after 2 rounds from 100 ng total RNA template; considered failed
5/21/01	continue one additional round and re-analyze	Ribogreen Assay and RT-PCR	using an aliquot of material already amplified 2 rounds, yield of cRNA was 80 ng (no increase); RT-PCR tests OK for GAPDH and Actin, but not high MW transcripts or PolE 606 (low expression)
6/12/01	first attempt using tailed LASS procedure modified for magnetic bead solid-phase synthesis	RiboGreen Assay	2 rounds of amplification appeared to produce 4-7 µg cRNA from 100 ng template

6/25/01	retest solid-phase LASS for product quality	radiolabeled RNA gel electrophoresis	procedure appeared to work; smear of products ranging from 400-4000 bp; biased towards heavier products as expected and compares favorably to size distribution of parent RNA;
6/28/01	performed solid-phase tailed LASS procedure using 100 ng total RNA; samples submitted to UVA Affymetrix Facility for microarray analysis	RiboGreen Assay+	assay shows yield of 13-17 µg (within desired target range); parallel sample submitted for Affymetrix analysis which indicated that no specific transcripts were present in the amplified material
7/6/01	tests of size distribution of modified Eberwine aRNA method	radiolabeled RNA gel electrophoresis	tested after 2 rounds of amplification; size distribution concentrated at or below 500 bp, which indicates significant problems with full-length product
7/24/01	pilot tests of aRNA method by Watson, et.. al.; direct comparison to "best" Eberwine-derived protocol to date using 100 ng total RNA template	Agilent Bioanalyzer; Affymetrix GeneChip	cDNA from 1 round T7 amplification submitted; yields of biotin-labeled material were 17.9 µg for the Watson prep and 0.7 µg for the Eberwine prep; Watson yield was ≥10 µg, so was sufficient to test on a GeneChip. Results were consistent with other doubly--amplified material.
7/31/01	tests of Watson method and three modified Eberwine methods using 50 ng total RNA template. Changes to Eberwine are a) use of spin columns in same fashion as Watson method b) use of Qiagen Sensiscript enzyme for cDNA preparation c) elimination of all spin column steps	RiboGreen Assay	performed 2 rounds of T7 amplification and quantitated (equivalent to mock-biotin labeling); Watson yield 12.5 µg; Sensiscript yield 0.4 µg; Eberwine spin col. 11.9 µg; Eberwine alcohol ppt 11.3 µg
8/30/01	retests of improved aRNA methods for microarray analysis; modified Eberwine procedure, replicate modified Watson procedure, Watson with overnight T7 synthesis performed on 50 ng total RNA template to assess performance in biotin labeling and microarray	Agilent Bioanalyzer; Affymetrix GeneChip	biotin labeled cRNA yields were Eberwine 0.9 µg; Watson 1 3.4 µg; Watson 2 0.5 µg; Watson O/N 14.0 µg. Only Watson O/N gave sufficient material for a GeneChip test. Background was abnormally high and gene detection was low for this sample. Concluded that 50 ng (5000 cell equiv) may be close to lower limit for amplification methods.
9/20/01	input template titrations of modified Watson aRNA procedure. Prepared duplicate samples using total RNA template equivalent to 5000, 10000, and 20000 cells. One of each pair used standard 4 hour T7 in-vitro transcription (IVT) and one used overnight IVT	Agilent Bioanalyzer; Affymetrix GeneChip	biotin labeled cRNA yields were 5K 3.1 µg; 5K O/N 3.7 µg; 10K 2.7 µg; 10K O/N 6.3 µg; 20K 2.8 µg; 20K O/N 7.4 µg. Only O/N 10K and 20K sufficient for array analysis. Both samples showed significantly higher than normal 3-prime bias,and background hybridization was very high for 10K cell sample.
10/3/02	tests of new RNA isolation kit (Stratagene Nanoprep) which is a rapid spin-column isolation designed for use with very small cell numbers. Tested with range of total RNA inputs and with 10,000 and 20,000 microdissected prostate cells	RiboGreen Assay	recovery efficiency was below 20% for total RNA inputs in desired target range of 100-200 ng. Microdissected cells preformed better, but yields were only 50% of expected from number of cells used

	additional tests with microdissected prostate cells and small quantities of input to verify previous results and to compare our previously validated Stratagene MicroRNA isolation procedure and optimized DNase treatment procedure	RiboGreen Assay	our previously validated procedure gave 30% better yields than the Nanoprep kit for total RNA, and recovery of RNA using microdissected material from the Nanoprep kit was well below expected amounts from number of cells used; conclusion is that kit is not useful.
12/4/01	performed tests using integrated commercial kit for RNA amplification (Ambion MessageAmp Kit)	RiboGreen Assay	first test of kit failed outright due to faulty kit instructions regarding incubation conditions (0.36 µg yield);
12/17/01	performed tests of spin columns (Qiagen RNeasy) versus PCI extraction/alcohol precipitation for recovery of cRNA after amplification and preliminary cleanup prior to DNase treatment. Necessary to identify whether salt carryover or SpeedVac steps required	RiboGreen Assay	Using 20,000 microdissected prostate cells, the use of spin-column for initial purification gave widely varying results (6 µg to 19 µg at end of procedure). PCI extraction gave cRNA yields of 21 µg, which is more than sufficient for arrays
1/23/02	further tests using Ambion MessageAmp Kit after modifications to supplied protocol to correct previously observed deficiencies	RiboGreen Assay	adjustments to protocol to correct this were performed and tests re-tried using 200 ng total RNA input with resulting yield after mock biotin labeling of 11.6 µg
2/19/02	further tests using Ambion MessageAmp Kit using 200 ng total RNA (20,000 cell equivalents) versus best Watson procedure to date	Agilent Bioanalyzer; Affymetrix GeneChip	performed biotin labeling using procedure from kit on 1 sample (6 µg yield); submitted 1 sample to UVA Affymetrix Core for their standard biotin labeling (7 µg yield). Both Biotin samples analyzed on Affymetrix Test Chips along with one modified Watson sample (10 µg yield).
3/1/02	Tests of RNA isolation methods for microdissected prostate material under Leica laser microdissection conditions using newly available Agilent Bioanalyzer instrument. Compared Qiagen RNeasy isolation to 2 variations on Stratagene MicroRNA isolation protocol.Also tests of newly available Zymo Research RNA isolation kit. Parallel tests to evaluate effect of DNase treatment steps on quality and yield of RNA	Agilent Bioanalyzer	Zymo kit found not to be compatible with mineral oil used in Leica LMD samples. Stratagene MicroRNA isolation buffer found to be problematic with spin-column purifications. "Standard" Stratagene MicroRNA conditions verified as best RNA isolation procedure.
4/17/02	Compared replicate procedures: new Affymetrix aRNA protocol and modified Watson protocol. IVT conditions were lowered from overnight back to 4 hours in order to reduce background seen from overproduction of aborted transcripts in our earlier experiments. Used revised RNA isolation procedure for all. Input material was approximately 10,000 cells from frozen prostate histologic section.	RiboGreen Assay; Agilent Bioanalyzer; Affymetrix GeneChip	After one T7 amplification round, Affymetrix method produced 0.2 and 0.3 µg cRNA. Improved Watson procedure produced 1 µg and 0.7 µg. Remaining Watson material was converted to cDNA and submitted for biotin labeling and GeneChip analysis, along with unamplified material from same frozen tissue source. Biotin labeled Watson preps produced 78 µg and 72 µg respectively. The amplified samples and RNA directly from the source tissue were then subjected to GeneChip analysis. The microarray results from this experiment showed similar results for the amplified material compared to the source tissue, with very low background.

The results from our optimization, just completed, is that we can obtain in the range of 70 ug of amplified, biotin-labeled cRNA starting from 100 ng of total RNA. This corresponds to between 10^5 and 10^6 fold amplification of mRNA after 2 rounds of in vitro

transcription reactions. Table 2 shows some results from GeneChip hybridization, comparing amplified RNA obtained from the procedures described in Table 1 under 4/17/02 to the results from unamplified RNA obtained from the same tissue. The results show similar levels of background, number of genes identified and similar 5' to 3' bias between amplified and unamplified RNA. These results show us that we have the procedures in place to now apply GeneChip analysis on microdissected samples. Our latest RNA amplification protocol is present in the appendix.

Table 2: Affymetrix GeneChip Hybridization Quality Data

sample	BG	%P	GAPDH 3'/5'	b-actin 3'/5'
non-amplified 1	99.2	24.1	8.3	15
non-amplified 2	117.2	27	9.5	26.8
non-amplified 3	91.1	24.9	6.4	16.2
amplified 1	106	22.6	9.2	21.2
amplified 2	122.6	22	9.1	22.1

BG: background levels of hybridization, %P: number of genes on array recognized as present in hybridized RNA, GAPDH 3'/5': ratio of hybridization to 3' and 5' array features of GAPDH gene, b-actin 3'/5': ratio of hybridization to 3' and 5' array features of beta-actin gene.

We are now ready to apply this technique to target tissues to study gene transcription changes during the neoplastic transformation in prostate epithelium. At the time of this report, we have microdissected two 10,000 cell aliquots of prostate epithelium. Our target is 10 cases each of benign prostate epithelium, high grade prostatic epithelium and invasive prostate cancer. The microarray to be employed is the U95a Affymetrix gene chip.

Phase II: Due to the lack of success in the implementation of representational difference analyis and differential subtraction chain techniques, the original phase 2 was not implemented. The remainder of the grant period was spent in developing the appropriate techniques for RNA amplification in order to perform microarray analysis on microdissected tissue.

Key research accomplishments

- Optimization of RNA amplification procedures to allow microdissected prostate cancer samples to be used on oligonucleotide microarrays.
- Establishment of laser assisted microdissection techniques for the collection of pure populations of prostate cancer, prostatic intraepithelial neoplasia, benign prostate epithelium and surrounding stroma.

Reportable Outcomes

The results of this work have formed the basis of a grant application to the Paul Mellon Prostate Cancer Research Institute Research Opportunities Fund, Title: *Microgenomics approach to the analysis of human prostate cancer tumor progression.* Specific Aims: 1) Validate an established RNA amplification protocol for representative gene expression

profiling of microdissected cell populations obtained from clinical human tissue samples. 2) Identify gene expression patterns that identify the transition from prostatic intraepithelial neoplasia to invasive prostate cancer.

Conclusions: Our studies have underscored the need for purification of pure representations of prostate cancer cells for global assays of genomic loss and for gene expression profiling. In these studies we have employed three different microdissection technologies (hydraulic micromanipulator, laser capture microdissection, laser assisted microdissection), and are convinced that pure representations of target tissues can be obtained with all these methods. However, our studies have also underscored the difficulties inherent to dealing with the miniscule sample size that is obtained from microdissection. With representational difference analysis and differential subtraction chain technologies, in multiple attempts we were unable to reproducibly isolate expected differences from the genomes of prostate cancer samples, or from "spiked" controls using this low level of DNA as a starting point. These procedures were originally designed to start with microgram quantities of DNA, which are amounts that cannot practically be obtained from microdissection. We believe that the increased number of PCR amplification cycles required to obtain amplicon populations from these samples leads to shifts in the amplicon population, resulting in unpredictable, stochastic loss of the rare amplicons representing areas of deletion in the entire genome. In shifting techniques to looking for gene expression differences using RNA transcript profiling on microarrays, our own experience, and the experience of others in profiling non-microdissected tissue samples is that the impure cell populations found in samples of cancer leads to the identification of spurious transcription patterns that are not reflective of the cancer cell transcript profile. Again, microdissection will eliminate this problem, but a major hurdle to overcome is the reproducible amplification of mRNA from microdissected samples. Polymerase chain reaction was avoided due to the non-linear amplification that tends to occur with this technique, and to avoid problems similar to those encountered with RDA and DSC. Coupled cDNA synthesis and in vitro transcription reactons ("aRNA") has been found to amplify mRNA transcripts from as little as 100 ng of RNA (10,000 cells) to quantities sufficient to detect the same numbers of genes on microarrays that can be detected with non-amplified RNA.

Although the official reporting period for this effort has ended, we are just starting the application of these methods on a set of clinical samples. Although we realize that this will fall out of the time limit for reporting results, we will nevertheless report to you any successful studies based on the methodology developed during this grant, and will acknowledge the support of this DAMD grant in any future publication resulting from this work.

List of personnel with salary supported by grant: Sue Rutherford Ph.D. (post-doctoral fellow), Craig Rumpel M.S. (senior laboratory specialist).

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Appendix

aRNA protocol

Protocol Notes:

- Use nuclease-free water for all steps. Do not use DEPC-treated water or reagents made with DEPC-treated water for this procedure. RNA which has been chemically modified by DEPC cannot be used as a transcription template.
- Preferred T7 primer sequence is the standard oligo recommended by Affymetrix for Enzo T7 synthesis
  [5'–ggCCAgTgAATTgTAATACgACTCACTATAgggAggCggT(24)–3']
- Set up reactions in sterile, RNase-free 0.65 mL tubes, and use a sub-ambient capable thermal cycler with heated lid for all incubations. Water baths are a haven for fungi, and fungal/mildew RNases are not affected by enzymatic blocking reagents. Use of the hotlid is essential for all but sub-ambient incubations. Use the tube thermistor for temperature control of the PCR machine.
- Do not use glycogen carrier at any point in the procedure, as this interferes with small-scale cDNA synthesis procedures.

RNA isolation

1. Stain flash-frozen, EtOH fixed 10 µM tissue sections with per standard protocol.
2. Microdissect approximately 10,000 cells using standard techniques for the Leica LMD instrument.
3. Spin down the microcentrifuge tube containing the microdissected material and oil used to aid capture for 5 minutes (RT) in a benchtop microcentrifuge.
4. Add 100 µL RNA denaturing buffer (Stratagene) and 0.72 µL b-mercaptoethanol.
5. Vortex to bring all the tissue pieces into solution, and continue to incubate with the denaturing buffer for 2 minutes. Continue to gently vortex the samples throughout this incubation to ensure complete tissue denturation. Bump the samples down in a microcentrifuge and transfer to a clean 0.65 mL tube.
6. Add 10 µL (0.1X volume) 2M sodium acetate (pH 4.0; Stratagene)
7. Add 110 µL (1X volume) acidic, water-saturated phenol (bottom layer; Stratagene) and 30 µL (0.3X volume) chloroform-isoamyl alcohol (24:1; Stratagene).
8. Vortex vigorously for 30 seconds, then incubate at 4°C for 15 minutes to maximize extraction. Use a cold tube block for this step.
9. Centrifuge for 10 min at 14,000 x g (RT) to separate the aqueous and organic phases.
10. Transfer upper aqueous layer to a new 0.65 mL tube
11. Add 4.0 µL of 5 µg/µL Linear Acrylamide (Ambion).
12. Add 110 µL (1X volume) isopropanol
13. Freeze solid in dry ice/ethanol bath (approximately 10 minutes). Alternatively, the tube may be left at -80°C overnight and then proceed from this point the next day.
14. Centrifuge for 30 min at 16,000 x g (4°C), with caps hinges pointing outward so that the location of the pellet can be better predicted
15. Remove the majority of the supernatant while attempting to minimize disruption of the RNA pellet. The pellet may not be visible at all. It is preferable to use a 200 µL pipette tip, instead of a larger one to avoid aspirating it.
16. Wash with 400 µL 70% ethanol (4°C). Centrifuge for 5 min at 16,000 x g (4°C)

17. All of the supernatant should be removed at this point. Use care when pipetting as pellets will be very loosely adhering to the tube. It should become visible at this point but will be small.
18. Let the pellet air dry briefly (≤5 minutes) in the PCR hood to remove any residual ethanol.
19. Pellet can be briefly stored in the -20°C PCR freezer until further use or stored for longer periods at -80°C. RNA pellets are most stable for storage when not reconstituted

cDNA Synthesis Round 1 from total RNA

1. Mix total RNA sample with T7-polydT primer as follows:

total RNA (in nuclease-free dH_2O)	11.0 µL
100 µM T7-polydT primer	1.0 µL
total volume	12.0 µL

2. Incubate at 70 °C for 10 minutes (hotlid on) and then cool to 4°C in the PCR machine.
3. Transfer to ice, add the following to the above mixture in the order specified:

5X First Strand Buffer (Life Technologies)	4.0 µL
0.1M DTT	2.0 µL
10 mM dNTPs	1.0 µL

Mix, incubate at 42 °C for 2 minutes, and then add:

Superscript II (Life Technologies; 200 U/µL)	1.0 µL
total volume	20.0 µL

Mix and incubate at 42 °C for 1 hour (hotlid on).

4. Spin the first-strand synthesis reaction down, place on ice, and add the following for second strand cDNA synthesis (hold components on ice after thawing and assemble cold):

nuclease-free dH_2O	91.0 µL
5X Second Strand Buffer (Life Technologies)	30.0 µL
10mM dNTPs	3.0 µL
E.coli DNA polymerase I (10 U/µL)	4.0 µL
E.coli DNA ligase (10 U/µL)	1.0 µL
RNase H (2 U/µL)	1.0 µL
total volume	150.0 µL

Mix and incubate at 16 °C for 2 hours in the PCR machine (hotlid off).

5. Add 2.0 µL of T4 DNA polymerase (5 U/µL) and incubate at 16 °C for 5 minutes.
6. Stop the reactions by adding 25 µL 0.2 M EDTA. Samples may be stored at -20°C or proceed immediately to cleanup. The cDNA will not be in buffered solution after cleanup, so T7 synthesis must be performed directly afterward.
7. PCI extract as follows using Phase Lock Gel (Eppendorf)(0.5 mL tubes, "heavy" gel formula):
   a) Centrifuge Phase-Lock Gel tube for 30 seconds at 14,000 x g.
   b) Add 177 µL (1 vol.) phenol:chloroform:isoamyl alcohol (pH 8.0) to the cDNA reaction.
   c) Vortex for 30 seconds.
   d) Transfer to the Phase-Lock Gel tube (do not vortex this tube).
   e) Spin at 14,000 x g for 5 minutes (RT).
   f) Transfer the aqueous phase (upper) to a fresh 1.5 mL tube, noting the volume obtained.
   g) Add 0.5 volumes (~80 µL) 7.5 M ammonium acetate, mix well, and add 2.5 volumes (~400 µL) 100% ethanol.
   h) Vortex to mix and centrifuge immediately for 20 minutes at 16,000 x g (RT). Immediate centrifugation of the ethanol-precipitated cDNA and washing are critical for avoiding carryover of ammonium salts which inhibit T7 synthesis.

i) Wash the pellet two times with 500 µL 70% ethanol, spinning for 5 minutes at 16,000 x g (RT) each time. Pellet may not be visible. Carefully remove as much of the supernatant as possible after the last wash.
j) Allow the pellet to air dry briefly and resuspend in 8 µL nuclease-free dH_2O.

T7-In Vitro Transcription (Ambion MEGAscript T7 Kit)

1. Add the following in the order specified to the 8.0 µL cDNA obtained above:

10X MEGAscript Reaction Buffer	2.0 µL
ATP solution	2.0 µL
CTP solution	2.0 µL
GTP solution	2.0 µL
UTP solution	2.0 µL
MEGAscript T7 Enzyme Mix	2.0 µL
total volume	20.0 µL

Mix well by pipetting and incubate at 37 °C for 18 hours in the thermal cycler (hotlid on). Hold at 4 °C when complete. Samples should be processed as soon as possible after completing the incubation. Do not exceed the 18 hour incubation time and do not hold reactions at 4°C for more than a few minutes.
2. Purify using QIAGEN RNeasy columns as described below:
   a) Add 80 µL water to bring reaction volume to 100 µL and transfer to a clean 1.5 mL tube.
   b) Add 350 µL Buffer RLT (prepare an aliquot by adding 10 µL β-mercaptoethanol per 1 mL of Buffer RLT; stable at 4°C for 30 days).
   c) Add 250 µL absolute ethanol. Mix thoroughly and transfer to an RNeasy column.
   d) Spin at 12,000 x g for 15 seconds.
   e) Discard the flowthrough and add 500 µL of Buffer RPE.
   f) Spin at 12,000 x g for 15 seconds.
   g) Discard the flowthrough and add 500 µL of Buffer RPE
   h) Spin at 12,000 x g for 1 minute.
   i) Transfer column to a new catch tube (provided with the kit) and spin for 1 minute at 12,000 x g to remove any residual buffer.
   j) Transfer column to a 1.5 mL tube and add 50 µL of nuclease-free water to the column. Add the water directly to the center of the membrane and spin at 12,000 x g for 1 minute. Add 30 µL additional nuclease-free water and spin at 12,000 x g for 1 minute, giving a total of 80 µL eluate.
3. Immediately alcohol precipitate the aRNA as follows:
   a) Add 0.5 volumes (~40 µL) 7.5 M ammonium acetate, 4 µL 5 µg/µL linear acrylamide (Ambion), and 2.5 volumes (~200 µL) 100% ethanol.
   b) Vortex to mix and centrifuge immediately for 30 minutes at 16,000 x g (RT).
   c) Wash the pellet two times with 500 µL 70% ethanol, spinning for 5 minutes at 16,000 x g (RT) each time. Pellet may not be visible. Carefully remove as much of the supernatant as possible after the last wash.
4. Allow the pellet to air dry briefly and resuspend in 11.0 µL nuclease-free dH_2O. If not continuing immediately to cDNA synthesis, leave the pellet dry and store at -70°C.

cDNA synthesis Round 2 from Amplified RNA

1. To the 11.0 µL cRNA from above add 1.0 µL of 1 µg/µL random hexamer primers (total volume 12.0 µL).
2. Incubate at 70 °C for 10 minutes (hotlid on), then cool to 4 °C. Spin down briefly and place sample on ice.
3. Add the following to the mixture above in the order specified:

5X First Strand Buffer (Life Technologies)	4.0 µL
100 mM DTT	2.0 µL
10 mM dNTPs	1.0 µL

   Mix, incubate at 42 °C for 2 minutes, then add:

Superscript II (Life Technologies; 200 U/µL)	1.0 µL
total volume	20.0 µL

   Mix and incubate at 42 °C for 1 hour (hotlid on).
4. Add 1.0 µL of RNase H (2 U/µL) and incubate for 20 minutes at 37 °C, heat to 95 °C for 2 minutes and cool to 4°C. Spin down briefly and place sample on ice.
5. Add 1.0 µL of 100 µM T7-polydT primer. Incubate at 70° C for 10 minutes, spin down briefly, then place sample on ice.
6. Add the following for second strand synthesis (hold components on ice after thawing and assemble cold):

nuclease-free dH_2O	90.0 µL
5X Second Strand Buffer (Life Technologies)	30.0 µL
10mM dNTPs	3.0 µL
E.coli DNA polymerase I (10 U/µL)	4.0 µL
E.coli DNA ligase (10 U/µL)	1.0 µL
total volume	150.0 µL

   Mix and incubate at 16 °C for 2 hours (hotlid off).
7. Add 2.0 µL of T4 DNA polymerase (5 U/µL) and incubate at 16 ° C for 10 minutes.
8. Stop the reactions by adding 25 µL 0.2 M EDTA. Samples may be stored at -20°C or proceed immediately to cleanup. Use the same procedure as before, except samples may be resuspended in 22.0 µL of nuclease-free water.
9. The cDNA will not be in buffered solution after cleanup, so T7 synthesis should ideally be performed directly afterward.